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MOLECULAR CHARACTERIZATION OF ATTENUATED JUNIN VIRUS VARIANTS

FINAL REPORT

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Junin virus, one of the few human pathogenic arenaviruses, is the etiologic agent of Argentine hemorrhagic fever (AHF). The clinical symptoms of AHF include hematologic, neurologic, cardiovascular, renal and immunologic alterations. The mortality rate may be as high as 30%, but early treatment with immune plasma reduces the fatal cases to less than 2%. In order to control the endemo-epidemics in the richest farming land in Argentina a collaborative effort conducted by US and Argentine Governments led to the production of a live, attenuated Junin virus vaccine. After rigorous biological testing in rhesus monkeys, the highly attenuated Junin virus variant named Candid #1 (CD1) was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area. In order to characterize the vaccine strain CD1 at the molecular level and initiate studies on the biochemical basis of attenuation of virulence, the structural protein genes of this attenuated virus were cloned and sequenced. In addition, cDNA clones of the XJ#44 strain -a very close predecessor of CD1- were also analyzed. Several changes in the amino acid sequence of N were observed that alter both the net charge and the predicted secondary structure of this polypeptide. When the attenuated strains XJ#44 and CD1 were compared to the wild type MC2 strain, major changes in the amino acid sequence were observed in the amino terminal region of glycoprotein precursor gene (GPC) as a result of several insertions and deletions in the nucleotide sequence. After proteolytic cleavage of GPC these alterations appear in the G1 polypeptide, that is thought to be located on the surface of the virion in association with the more internal G2 protein. The predicted secondary structures of CD1 and XJ#44 G1 proteins are similar to each other. On the contrary, the G2 protein of CD1 has a different hydrophobic motif from those of XJ#44 and MC2, which bare a close resemblance to each other. The data on a more virulent predecessor of XJ#44 and CD1 viruses (e.i., XJ#13) should shed more light on the relevance of the genotypic changes reported here in relation to the molecular basis of the attenuation of virulence. The biological significance of the sequence changes will have to be assessed by developing a system for the regeneration of infectious virus from recombinant cDNA clones.

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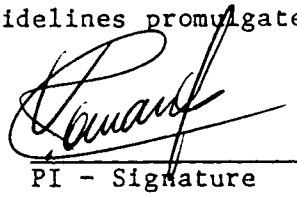

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CONTENTS

EXCERPT FROM THE RESEARCH PROPOSAL (June 1988)

The characterization of Junin virus **Candid #1 genome** is the primary goal of the present research proposal. Modern **nucleotide sequencing** technology provides a means of precise description of any genome. However, the sequence analysis of Candid #1 alone will not provide any clue to the mechanisms of virulence attenuation. Therefore, it will be necessary to compare it to sequences of **more virulent ancestors** in the passage history leading from prototype Junin virus to Candid #1 vaccine.

The availability of these genealogically related Junin virus variants (i.e. Candid #1, XJ#44, XJ#13) permits a proper approach to the issue of the molecular basis of arenavirus virulence.

This project focuses mainly on the **changes in the S RNA species**, since a plausible working hypothesis is that changes in tissue tropism affecting neurovirulence could possibly be related to changes in the viral **surface glycoproteins** encoded in this RNA segment. Further progress of this research will provide nucleotide and aminoacid sequence data pointing at molecular changes that will hopefully correlate with differences in virulence. Highly variable regions will also be highlighted.

The comparison of the sequences of the "avirulent" Candid #1 to more than one "virulent" ancestor will also allow for an estimation of the genome stability or the **probability of reversion** to the "virulent" phenotype. This is a question relevant to the theoretical estimation of the risk of using Candid #1 as a vaccine, although the **phenotypic stability** of it has been already assessed empirically.

In addition, the molecular analysis of the viral nucleic acid can be used as a part of a thorough **quality control** of vaccine preparations.

In the frame of the extended AHF vaccine program, a careful evaluation of the clinical and subclinical AHF cases must include the **genotypic identification** of the viruses **isolated from the patients** in the endemic area. Nucleic acid sequence analysis is the unambiguous piece of data that can tell us whether the disease is caused by an indigenous virus or a virus derived by mutation from the vaccine strain. The amplification of nucleic acids using the polymerase chain reaction (PCR) could be employed for this purpose based on the nucleotide sequence data generated in this project. This particular technology is being tested in order to evaluate its potential as a laboratory diagnostic assay.

In summary, the present project shoud be regarded as a first step in the identification of regions in Junin virus genome related to a particular virulence pattern. The other major objective is to provide a molecular description of the AHF vaccine strain.

INTRODUCTION

Junín virus is the etiological agent of Argentine hemorragic fever (AHF). The clinical symptoms of AHF include hematologic, neurologic, cardiovascular, renal and immunologic alterations. The mortality rate may be as high as 30%, but early treatment with immune plasma reduces the fatal cases to less than 2%. (Maiztegui *et al.*, 1979; Enria *et al.*, 1986).

A wide variation in annual number of cases occurs, ranging from 100 to 4000. Since its recognition in the early 1950s, the disease has spread from an area of 16.000 km^2 to an area greater than 120.000 km^2 of the richest farming land in Argentina, with a population of *ca.* 2 million inhabitants (Maiztegui *et al.*, 1986). The major rodent hosts for Junín virus are *Calomys musculinus* and *Calomys laucha* (Maiztegui, 1975), although infection has been detected on occasion in *Mus musculus*, *Akodon azarae* and *Orizomys flavescens* (Sabattini *et al.*, 1977). The human population at risk is composed mainly by field workers. Humans are believed to become infected through cuts or skin abrasions or through airborne dust contaminated with urine and saliva of infected rodents. A high degree of variation in virulence and clinical patterns has been reported for different isolates of Junín virus (Maiztegui, 1975; Mc Kee *et al.*, 1985; Kenyon *et al.*, 1988).

A collaborative effort conducted by US and Argentine Governments led to the production of a live, attenuated Junín virus vaccine (Peters *et al.*, 1987). After rigorous biological testing in rhesus monkeys (Mc Kee *et al.*, 1984), the highly attenuated Junín virus variant named Candid #1 was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area (Maiztegui *et al.*, 1990).

Our interests are directed towards the characterization of the vaccine strain at the molecular level. In addition, the availability of several genealogically related Junín virus variants with an increasing

degree of attenuation (e.g. XJ #13, XJ #44 and Candid #1), derived in the process of developing the AHF vaccine, permits a systematic approach to the issue of the molecular basis of Junín virus virulence.

In the last few years a reasonable amount of information has been accumulating related to the genome structure of some members of the Arenaviridae family, to which the Junín virus also belongs.

All the arenaviruses share morphologic and biochemical properties; they are enveloped and their genome consists of two single-stranded RNA species, designated L (for large, *ca.* 7 kb) and S (for small, *ca.* 3.5 kb). The L RNA codes for two proteins: a large L polypeptide, presumed to be the RNA polymerase, and a small zinc finger-like protein (Salvato and Shimomaye, 1989; Salvato *et al.*, 1989; Iapalucci *et al.*, 1989a, 1989b). The S RNAs of several arenaviruses have been sequenced and shown to have several common features. The nucleocapsid protein gene N and the precursor of the envelope glycoproteins GPC are encoded in an ambisense manner (Bishop, 1988).

The N protein (*ca.* 63 kDa) is translated from a viral-complementary or anti-genome-sense mRNA species complementary to the 3′ half of the viral S RNA (Auperin *et al.*, 1984a, 1984b; Romanowski and Bishop, 1985; Clegg and Oram, 1985; Franze-Fernández *et al.*, 1987). The GPC protein (*ca.* 57 kDa in the unglycosylated form) is translated from a viral or genome-sense mRNA corresponding to the 5′ half of the viral S RNA (Auperin *et al.*, 1984c; Romanowski *et al.*, 1985; Auperin *et al.*, 1986; Franze-Fernández *et al.*, 1987).

Recently, the complete nucleotide sequence of Junín virus S RNA has been determined in our laboratory. The information has been obtained from cDNA clones derived from the MC2 strain, a wild type Junín virus of intermediate virulence (Ghiringhelli et al., 1989; Ghiringhelli et al., 1991). These data were instrumental for the molecular characterization of

the attenuated variant of Junin virus named Candid #1. Here we report the complete sequence of the structural protein genes N and GPC of the attenuated Junin virus vaccine strain, Candid #1, and its close relative XJ#44 (Fig. 1). Their relationship to the wild type Junin virus MC2 and other closely and distantly related arenaviruses are also examined.

MATERIALS and METHODS

Virus and cell lines

The Junin virus vaccine strain, Candid #1 was kindly provided by Drs. J.I. Maiztegui and A.M. Ambrosio from the INEVH (Pergamino, Argentina) and propagated in certified fetal rhesus lung diploid cells (FRhL-2) at passage levels 19 to 28. A "working stock" of Junin Candid #1 virus was produced by infection of FRhL-2 cell monolayers with the master seed (Barrera Oro ARG 8/009, 14.8.81) at an m.o.i. of 0.01 PFU/cell. The supernatant media were harvested 96 h post infection, aliquoted and stored at -70°C. This virus stock was used as inoculum to produce virus particles for biochemical studies.

The attenuated Junin virus XJ#44 was provided by Dr. J.G. Barrera-Oro and cultured in our laboratory in BHK21c13 cells.

Preparation of viral RNA

When 50% confluent, FRhl-2 cell monolayers were infected with the Candid #1 strain (m.o.i. 0.1 pfu/cell) and incubated at 37°C in Eagle's minimal essential medium with non-essential amino acids (MEM) and 10% fetal calf serum. The virus was recovered and purified from the supernatant media on day 4 post-infection (Grau *et al.*, 1981, Rosas *et al.*, 1988).

The XJ#44 virions were recovered from the supernatant media of infected BHK21c13 cell monolayers on days 4 and 5 post-infection.

Virions pelleted by ultracentrifugation were disrupted with guanidinium thiocyanate and the RNA was isolated essentially according to the procedure of Chomczynski and Sacchi (1987). The RNA pellet was resuspended in 0.5% sarkosyl, phenol extracted and reprecipitated with 0.3M sodium acetate and 2.5 volumes of ethanol. The final pellet was resuspended in water and stored in aliquotes at -70°C.

The RNA was analyzed by agarose gel electrophoresis in the presence of methylmercury(II) hydroxide as denaturant (Bailey and Davidson, 1976).

Synthesis and cloning of cDNA

Synthetic oligonucleotides complementary to nucleotides 1-19, 846-862 and 2300-2316 (nucleotide numbers start at the 3′ end) of Junin virus MC2 S RNA (Ghiringhelli et al., 1989, 1990) were phosphorylated and used to prime cDNA synthesis. The reaction containing viral RNA, primers, deoxynucleoside triphosphates and AMV reverse transcriptase was carried out in a way similar to that described by Gubler and Hoffman (1983).

The double stranded cDNA was blunt-ended using T4 DNA polymerase, size-fractionated by gel electrophoresis and glass powder elution to prevent the generation of a high proportion of clones with short inserts. The ds cDNAs in the range of 1.0 - 4.0 kbp were inserted by ligation in the SmaI site of pBS SK$^-$ (Bluescript SK$^-$, Stratagene, La Jolla, CA) and cloned in *E. coli* DH5 F′α. The colonies containing pBS SK$^-$ with inserts were selected by color on LB-agar plates containing ampicillin, IPTG and X-gal.

Alternatively, Bst XI adapters were ligated to the ds cDNA prior to the size-fractionation step, and the cDNA was inserted in the Bst XI site of the pcDNA II vector (Invitrogen, San Diego, CA).

The screening of the recombinant clones was performed by colony hibridization (Grunstein and Hogness, 1975) using Junín-MC2 S RNA probes. The probes consisted of selected DNA fragments that were obtained from recombinant plasmids containing Junín-MC2 sequences and labeled by nick translation. Plasmid DNA was prepared from the hybridization positive clones, analyzed by gel electrophoresis after restriction enzyme digestion and tested by Northern blot (Maniatis *et al.*, 1982).

PCR amplification and cloning

In order to increase the efficiency of the cDNA cloning and compensate for the low yields of viral RNA, selected regions of the S RNA were amplified using RT-PCR (Doherty et al., 1989).

Briefly, after first strand cDNA synthesis, the target sequence was amplified using two flanking primers and the thermostable DNA polymerase of *Pyrococcus furiosus* (*Pfu* DNA pol.) in a 35-cycle-reaction with an extension time of 3 minutes for 1.5 kb DNA fragments. The reaction conditions were according to the manufacturers recommendations (Stratagene, La Jolla, CA).

In some experiments designed to determine the 5′ most terminal sequence of the RNA, the first strand cDNA synthesis was primed with an oligonucleotide complementary to a region some 180-220 nucleotides downstream from the 5′ end (Figs. 2 and 3). The primer extension product was purified by gel electrophoresis and tailed with dCTP and terminal deoxynucleotyl transferase. This ss cDNA was PCR-amplified using the previous primer and an oligonucleotide containing an oligo dG sequence preceded by a Cla I and Eco RI recognition sequence ($GATCGATGAATTCG_8$).

The amplified DNAs were electrophoresed on an agarose gel in TAE buffer with 0.25 μg/ml ethidium bromide. The DNA bands were located under long wave UV illumination, excised and purified using glass powder and NaI

(BIO 101, La Jolla, CA). The ends of the DNA fragments were repaired with dNTPs and the Klenow fragment of *E. coli* DNA polymerase, and cloned by blunt end ligation into pUC19 (Yanisch-Perron et al., 1985).

Northern blot analysis

Viral and cellular RNAs were denatured with 10mM CH_3HgOH and electrophoresed on a 1% agarose gel according to Bailey and Davidson (1976). The gel was soaked in 14 mM β-mercaptoethanol and 0.5 μg/ml ethidium bromide, to check the quality of the RNA preparation, and transferred by capillary blotting onto a Gene Screen nylon membrane (NEN, Chicago, IL, USA). The blot was processed according to the manufacturers specifications and hybridized to different cDNA probes. The ^{32}P labelled cDNA probes were prepared by nick translation (Maniatis *et al.*, 1982) or multipriming (Feinberg and Vogelstein, 1983) of the recombinant plasmid DNAs. Northern blots were employed to assess the identity of the cDNA clones that were further analyzed by nucleotide sequencing.

Sequence analysis

The nucleotide sequences of the cDNA inserts were determined by the dideoxy method of Sanger *et al.* (1977) using a modified T7 DNA polymerase (Sequenase™, USB Cleveland, OH, USA). The original cDNA inserts were subcloned into M13 mp18 and mp19 and the ssDNAs were used as templates for sequencing (Yanish-Perron *et al.*, 1985).

The sequence information was processed on a personal computer using the DNASIS (Hitachi Software Engineering Co.) program and on a VAX computer using the package by Genetics Computer Group (GCG, Madison, WI, USA).

RESULTS

Molecular cloning and nucleotide sequence of Junín-Candid #1 virus S RNA

Different oligonucleotides were used simultaneously (Fig. 2) to prime cDNA synthesis on Junín-Candid #1 S RNA. The selected cDNA clones were shown to anneal specifically to Junín virus S RNA in Northern blot hybridizations (data not shown).

In addition to the standard cDNA synthesis and cloning procedures, several selected regions of the S RNA of Junín virus strains Candid #1 and XJ#44 were amplified by RT-PCR to improve cloning. In this way, full-length clones were generated for the N and GPC open reading frames (ORF), as well as, independent clones for the G1 and G2 regions of the GPC precursor. In addition, several PCR clones were isolated encompasing the noncoding sequences of the S RNA and overlapping the ends of the ORFs (Fig. 2 and 3).

The nucleotide sequences of several overlapping cDNA clones shown in Fig. 2 were determined using the dideoxy chain termination method (Sanger *et al.*, 1977).

The nucleotide sequences of the GPC and N ORFs of Candid #1 and XJ#44 strains are presented in figures 4 and 9, respectively, aligned with those of the MC2 strain of Junín virus (Ghiringhelli et al., 1991). The homologous regions of Tacaribe virus S RNA were included in the figures for comparison with this arenavirus closely related to Junín virus.

The noncoding regions of the S RNAs of these viruses are shown in figures 13 and 14.

Junín Candid #1 virus glycoprotein precursor GPC

The first AUG codon of the genomic or viral-sense (v) S RNA, found at nucleotides 89-91, initiates a long ORF that remains open down to the in-

phase UAA translational termination signal at positions 1544-1546. Seven codons downstream from the first AUG, a second AUG is found in the same frame. However, based upon the observations on most frequent flanking sequences of eukaryotic translation start codons reported by Kozak (1984) (i.e. CC$\overset{A}{G}$CCAUGG), the first AUG is considered to function as the true initiation codon since a G residue is found at positions +4 and -3, and there is also a C at position -2. The molecular weight calculated for the 485 amino acids long translation product (i.e. 55,700 Da) is smaller than the value estimated by gel electrophoresis for the intracellular glycoprotein precursor that undergoes cotranslational glycosylation (De Mitri and Martinez Segovia, 1985; Rustici, 1984).

Eight potential N-glycosylation sites are found in the GPC sequence (Fig. 5). Whether all are used for the attachment of carbohydrate side chains is not known.

Amino acid sequence homology of arenavirus GPC gene products

Junin virus GPC protein sequence was aligned with the rest of the arenavirus GPCs as depicted in Fig. 6. The best-fit alignment shows that the amino acid sequence homology is concentrated in two regions: the 53 residues at the amino-terminus and the carboxy-proximal half of GPC. Buchmeier *et al.* (1987) demonstrated that, in the infected cell, LCM virus GPC is cleaved at or near the Arg_{262}-Arg_{263} to yield the proteins G1 (amino-terminal half) and G2 (carboxy-terminal half). By analogy, Junin virus GPC should be cleaved at or near the homologous Arg_{243}-Arg_{244} (MC2) or Arg_{247}-Arg_{248} (Candid #1 and XJ#44).

The G1 and G2 regions of GPC defined by the dibasic amino acid sequence at positions 243 to 286 —depending on the virus— exhibit quite different degrees of sequence homology. G1 sequences show few clusters of

sequence conservation among all the different arenaviruses. These homologous regions concentrate especially at the positions 1 to 53 and 84 to 97.

This sequence comparison shows that Junín and Tacaribe G1 sequences are by far more homologous (50%) than the other pairs analyzed. The sequence similarity is even higher for the G2 polypeptide, where 82% of the amino acids are identical in nature and position. Comparison of Junín G2 with the other arenavirus G2 proteins also shows a high degree of homology, in the range of 53-57%, suggesting that there is less sequence flexibility than in G1, compatible with function conservation. The homology figures are higher when conservative amino acid changes are considered in the calculation (Ghiringhelli et al., 1991).

Four of the putative N-glycosylation sites of GPC are conserved in all the arenaviruses (one in the G1 region and three in the G2, Fig. 6). This fact, however, is not necessarily linked with the actual use of these or the other less conserved glycosylation sites.

Comparison of the glycoprotein genes
of Junín virus strains Candid #1, XJ#44 and MC2

The alignment of the coding sequences of MC2 and Candid #1 GPC genes shows an overall high degree of homology (Tables 1 and 2, Fig. 7). Although, all the nucleotide sequence alterations are summarized in Table 3, there are major changes that will be pointed out here. There are several nucleotide insertions and deletions concentrated between the codons 43 and 76 of Junín-MC2 GPC gene that should be formally considered to modify its sequence to that of Candid #1 GPC (codons 43-80, Fig. 4).

There are six single-nucleotide insertions that change MC2 amino acid sequence $_{43}$CSILD$_{47}$ into $_{43}$LFQFFVF$_{49}$ in Candid #1. Two triplets downstream,

there is a six-nucleotide-block insertion resulting in the insertion of two amino acids (LA, codons 52 y 53). Further downstream, one transversion and three single-nucleotide insertions change the amino acid sequence $_{54}PR_{55}$ to $_{58}TEE_{60}$. The other major change results from three different single-nucleotide deletions that shift the reading frame of the coding sequence converting the MC2 sequence $_{65}$VPDCVLLQWWVS$_{76}$ to $_{70}$FQTVSFSMVGL$_{80}$.

Most of the other nucleotide substitutions do not change the coding with the exception of codons 139, 423 and 442 in MC2 or corresponding codons 143, 427 and 446 in Candid #1, that result in three amino acid changes, two of which are conservative according to the considerations of Schwartz and Dayhoff (1979).

As shown in Fig. 7, the distribution of the amino acid sequence changes in MC2 and Candid #1 GPC gene products concentrates in the amino-proximal region of G1 in contrast with the changes in the nucleotide sequence that are scattered through the carboxyl-terminus of G2 (Fig. 4). However, most of the nucleotide substitutions in G2 do not alter the amino acid sequence (Fig. 8).

When XJ#44 GPC ORF is examined no nucleotide deletions/insertions are found with respect to Candid #1 GPC. The major sequence changes described when Candid #1 G1 region was compared to MC2 were also found for XJ#44 (Fig. 4).

Only one transversion (G-C) was observed when the sequence of one XJ#44 G1 clone was compared to the Candid #1 sequence. This change introduced one conservative amino acid substitution (V-L). Another fully sequenced XJ#44 G1 clone showed two transversions (G-C, C-G), that resulted in one conservative (I-M) and one non-conservative amino acid change (S-F).

On the other hand, the G2 region of XJ#44 does not contain the 19 nucleotide substitutions of Candid #1 (compared to MC2) making the sequence almost identical to that of the MC2 strain. There are, however, two

nucleotide transitions (C-T; C-T) in each of two XJ#44 clones in positions, where Candid #1 G2 ORF does not differ from that of MC2. Both mutations in each of the sequences result in non-conservative amino acid substitutions (i.e. F → S, C → R in clone 1; S → F, S → F in clone 2).

It might be interesting to note the overall high score of homologous amino acid sequences when Junín virus GPCs are compared to Tacaribe virus GPC (Tables 1 and 2). In addition, the sequence changes introduced by the series of insertions/deletions in Candid #1 and XJ#44 make the first 80 amino acids of their G1 proteins almost identical to the 80 N-terminal amino acids of Tacaribe G1 -a region that exhibits many sequence differences among the members of the Arenaviridae family (Fig. 6 and 7).

None of the amino acid residues that are different in the Junín virus strains alters any of the eight potential N-glycosylation sites.

Junin virus N gene of the attenuated Junín virus vaccine strain, Candid #1

Examination of the antigenomic or complementary S RNA sequence indicated that the first AUG, at positions 82 to 84 (CAU anticodon, nucleotides 3317 to 3319 in the vRNA sequence of Junín virus MC2 used as reference, Ghiringhelli et al., 1991) is most likely to be the true translation initiation codon. It starts a long ORF (Fig. 9) which terminates at a UAA stop codon at nucleotides 1775 to 1777 (UUA anticodon, nucleotides 1625 to 1627 of the vRNA sequence) and the flanking nucleotides (**CTGGCAUGG**) conform well to the optimal context for initiation identified by Kozak (1984). The primary translation product, for which no proteolytic processing has been reported, has a calculated molecular weight of 63,033 Da, in good agreement with the electrophoretic mobility of Junin virus N (Martinez Segovia and De Mitri, 1977; Grau *et al.*, 1981).

The net charge of this polypeptide is estimated from its amino acid

sequence to be +11.5 at neutral pH. The net positive charge is a reflection of the relative abundance of the basic amino acids K and R, which are found scattered throughout the primary structure of N. In addition, several clusters of two, three and four basic amino acids are noted with frequencies above average (Fig. 10).

Comparison of Junin virus and other arenavirus Ns

A best-fit alignment of Junin virus N protein with those of each of the other New and Old World arenaviruses that have been sequenced indicates a high degree of sequence conservation. Several regions of identical amino acid sequences conserved among all the arenavirus N proteins are readily seen in Fig. 11.

In the same comparison, the similarity between Junin and Tacaribe virus N proteins is conspicuous, with very long regions of identical sequences and overall similarity of 76%. If conservative amino acid substitutions are considered according to the method of Schwartz and Dayhoff (1979), the similarity increases to 87%; any other pair exhibits lower levels of similarity. However, the sequence conservation among all the arenavirus N proteins reflects its extensive immunological cross-reactivity. Some clusters of conserved amino acid sequences correspond roughly to residues 154 to 163, 194 to 217, 232 to 265, 295 to 317, 453 to 463 and 523 to 534 of Junin virus N polypeptide (Figs 10 and 11).

Of the basic amino acids K and R in the Junín virus N proteins, 37 are conserved in the other arenaviruses (48.7%). In particular, the stretch of four basic residues some 60 to 70 residues from the C terminus is conserved among all the arenavirus Ns. The only exception is the Tacaribe virus N protein sequence in which KKNKSK is found in the position at which KKKR or KKKK is found in the other arenaviruses.

Comparison of the N genes of Junín virus strains Candid #1, XJ#44 and MC2

The aligment of the N coding sequences of the three Junín virus strains shows no nucleotide deletion/insertion changes. Both, Candid #1 and XJ#44 N genes differ by only 3 nucleotides; two of the substitutions produce non conservative amino acid changes, whereas the third one does not change the coding. There are 13 nucleotide substitutions between XJ#44 and MC2, and 14 substitutions for the pair Candid#1-MC2 (Fig. 9). Only two of the substitutions in XJ#44 and three in Candid #1 are silent, whereas the rest introduce 10 amino acid changes (common to XJ#44 and Candid #1) with respecto to MC2 (Fig. 12). Some of the amino acid differences result in changes of the net electrical charge, i.e. MC2, XJ#44 and Candid #1 N proteins exhibit values of +14.5, +12.5 and +11.5, respectively.

Most of the amino acid differences between MC2 and Candid #1 and XJ#44 are clustered between positions 96 and 112 and make the N sequence of the latter attenuated strains more similar to Tacaribe virus N in this region (Fig. 12).

Non coding nucleotide sequences

The first data derived from three independent cDNA clones indicated that the untranslated upstream sequence for Junín-Candid #1 virus GPC gene comprised 88 nucleotides, 86 of which were identical to the homologous region in Junín-MC2 S RNA. The two only differences that were found consisted of an extra cytosine at the 5′ end of Candid #1 RNA and the substitution of an A for a G at position 38.

Further experiments, in which more clones of this region were examined (nucleotides 22 through 90) showed several nucleotide differences, including insertions or deletions and substitutions (Fig. 13).

On the contrary, the 3' termini of the strains MC2 and XJ#44 do not show any nucleotide changes. However, not many clones of this region have been sequenced to date.

The non-coding region downstream from the translation termination signals for both GPC and N genes (nucleotides 1544-1546 and 1638-1640 of v S RNA) contains two sets of self-complementary nucleotide sequences capable of forming two very stable hairpin-loop structures (Fig. 14).

The hairpin closest to the GPC stop codon is formed by 11 GC and 2 AU pairs (ΔG°= -39.0 kcal/mol, Tinoco *et al.*, 1973) and the one closest to the N stop codon is stabilized by 15 GC and 1 AU pairs (ΔG° = -57.2 kcal/mol. The nucleotide sequences of Junin-MC2 and Junin-Candid #1 are identical but for three nucleotides. Moreover, two of the nucleotide changes (C→U, A→C) fall outside the double hairpin-loop, and the third one (U→C) does not affect the thermodynamic stability of the secondary structure either, since it is located at the top of the first hairpin-loop (Fig. 13). One hairpin-loop structure has been found in the intergenic region of most of the arenaviruses that have been sequenced to date (Auperin *et al.*, 1984; Romanowski and Bishop, 1985; Auperin et al., 1986; Franze-Fernández *et al.*, 1987). In this context Junin virus S RNA has an unusual secondary structure.

DISCUSSION

The nucleotide sequence of the S RNAs of the attenuated strains Candid #1 and XJ#44 have been determined by cDNA cloning and dideoxy sequencing procedures, and compared to each other and to the wild type MC2 strain.

During these studies, special attention was devoted to avoid spurious genetic variations that could possibly obscure the changes relevant to the

attenuation of virulence. Taking into account that arenaviruses are notorious for variations in their biological properties based on passage history (Ahmed *et al.*, 1984), the biochemical studies were conducted on virions that had the same manipulations as the vaccine stock. Junín (Candid #1) virus was passaged in diploid FRhL-2 cells the same limited number of times as the vaccine in order to avoid genotypic variations that could arise from accumulation of mutations upon repeated passage or a different type of selection posed on the virus if a different cell substrate was used.

On the other hand, the XJ#44 stock used in the RNA preparations had a different passage history prior to its arrival in this laboratory and had not been subjected to cloning by terminal dilution as was the vaccine strain.

The information obtained from cDNA clones of the attenuated Junín virus vaccine has been compared with the homologous regions of Junín-XJ#44, Junín-MC2 and other arenavirus S RNAs and their gene products.

Are the changes in the glycoproteins responsible for the attenuated phenotype of Junín-Candid #1 vaccine strain?

The involvement of the different parts of the arenavirus genome and their gene products in the pathogenicity of a particular virus strain is not clear. In this research project several differences in coding sequences of the GPC genes of three strains of Junín virus were found.

The nucleotide sequence changes found in the GPC ORF when Candid #1 and MC2 S RNAs are compared, concentrate in the NH_2-proximal (20 nucleotide changes) and the COOH-proximal regions (19 nucleotide substitutions). However, from Fig. 7 and 8 it becomes apparent that the major amino acid residue changes resulting from the above mentioned nucleotide sequence

alterations are located in the NH_2-proximal region of GPC. This part of the coding sequence corresponds to the G1 protein, which —by analogy with LCM (Burns *et al.*, 1990)— is thought to be exposed on the surface of the virion, in association with the more internal G2 protein.

The amino acid sequence changes occur in a region that contains the proposed signal peptidase cleavage site (Burns et al., 1990). Therefore, the altered G1 amino acid sequence would correspond to the actual amino-terminus of this protein. By contrast, only two isolated amino acid residue substitutions are found in the G2 protein of Candid #1, despite the fact that the number of nucleotide changes in this region of the genome is similar to the one in the G1 sequence.

These two amino acid changes make the predicted secondary structure of Candid #1 G2 protein conspicuously different from those of MC2 and XJ#44 strains (Fig. 15). This hydrophobic region in Candid #1 G2 lacks the four *β-turns predicted* for the MC2 and XJ#44 proteins using the algorithm Garnier-Osguthorpe-Robson (GCG Package, version 7, 1991).

On the other hand, no obvious structural differences are apparent between the G1 proteins of the attenuated strains XJ#44 and Candid #1.

Taking into account that, according to recent data on LCM virus from Dr. Buchmeier's laboratory, four molecules of each G2 and G1 polypeptides associate to form a stalk-and-head spike inserted in the viral envelope, the changes in one or both proteins might possibly alter the early interaction of the virion with the host cell.

Although our results that suggest the involvement of the surface glycoprotein in the attenuation of virulence are preliminary, they are consistent with the reports on other viruses.

It should be noted that for each attenuated vaccine strain analyzed (poliovirus types 1 and 3 and yellow fever) suggestive or experimental evidence has been obtained for a role of altered viral surface protein in

attenuation (Nomoto *et al.*, 1982; Hahn *et al.*, 1987). As suggested by the literature on different viruses the amino acid substitution can have profound effects on virulence and tissue tropism (Spriggs and Fields, 1982; Spriggs *et al.*, 1983; Coulon *et al.*, 1983; Johnson *et al.*, 1986; Prehaud *et al.*, 1990).

In particular, the literature on viruses such as paramyxo and orthomyxoviruses indicates that tissue tropism and virulence in a particular host are dependent upon the proteolytic activation of a surface glycoprotein (Tashiro *et al.*, 1990; Deshpande *et al.*, 1987). Although, the cleavability of the arenavirus GPC has not been studied in relation to the infection process, we have not found any amino acid sequence changes at or around the cleavage site of Junin Candid #1 GPC. Therefore, it might be concluded that this region plays no role in the attenuation of Candid #1.

The biological significance of the amino acid changes found in the attenuated and wild type strains of Junin virus might be provisionally summarized considering that both attenuated strains of Junin virus (XJ#44 and Candid #1) show the same "attenuating" motif at the N-terminus of G1, but the most attenuated Candid #1 has an additional "attenuating" change in the secondary structure of G2.

Changes in the amino acid sequence of N are less striking than in G1 and G2

The overall sequence identity between XJ#44 and Candid #1 N proteins is 99.6%, and 98.2% between each of these attenuated strains and the wild type MC2.

The sequence differences are highlighted in figure 12 and lead to two analyzable structural effects: a change in the net electrical charge and changes in the secondary structure patterns. The latter ones can be grouped as "MC2-like" and "Candid #1-like" structural motifs. From this

perspective, MC2 N has 4 MC2-like motifs, XJ#44 N has 1 of them (around amino acid 47) and 3 Candid #1-like motif, and Candid #1 contains obviously all 4 own motifs (not shown).

On the other hand, the net charges, that change from +14.5 in MC2 to -11.5 in Candid #1, are the result of substituting acidic amino acid residues for hydrophobic residues. The significance of these differences is not known, but it is conceivable to hypothesize that a possible effect might occur at the level of protein-RNA interaction or a putative N-G2 or N-Z association. At this point it might be reminded that N has been proposed to be the transcription antiterminator and, therefore, changes in its interaction with the RNA might affect the regulation of the transcription/replication process (Romanowski, 1992). Alternatively or concurrently, some of the changes in the N sequence might have an influence on the virion assembly or disassembly processes by affecting N-RNA and/or N-G2 interactions.

Variability in the non-coding sequence

The examination of the nucleotide sequence of a series of clones encompassing the 5′ untranslated region (5′ UTR) of Junin virus S RNA has shown a number of nucleotide changes. The limited set of data shown in figure 13 leads to the conclusion that some positions might be less critical for the function than others.

In particular, regardless of the strain, the sequence of 3 A residues in positions 72-74 (nucleotide numbering according to S RNA sequence published by Ghiringhelli *et al.*, 1991) appears to be very prone to extension or shortening. This result might indicate a "stuttering" mechanism of the RNA polymerase at this sequence (Jacques and Kolakofsky, 1990).

By contrast, other sequences -especially in the coding regions- seem

to be rather invariant not only within one single strain but even when different strains are compared (Figs. 4 and 9).

Although some of the nucleotide sequence data were obtained form RT-PCR amplified RNA, the most adverse estimations of the error rate introduced by either the *Taq* DNA polymerase or the *Pfu* DNA polymerase do not account for the nucleotide changes observed (Kunkel and Eckert, 1989). In addition, similar and/or identical changes were noted in clones generated from independent amplification reactions.

These data strongly suggest that the sequences presented in figure 13 represent the true variant RNA molecules in the viral RNA population. In this respect, the error rate of different RNA polymerases has been well documented and shown to give rise to different "quasispecies" from one original genome (Holland *et al.*, 1982).

At this point, very little can be said about the biological significance of this finding. The extent and sequence specificity of the variation and the proportions of each variant in the original RNA population, as well as in the progeny virus have to be examined to understand the overall picture.

Confirmation of an unusual secondary structure in the intergenic region

The nucleotide sequence of Junin-MC2 virus S RNA showed a potential secondary structure in the intergenic region consisting of two consecutive hairpin-loops (Ghiringhelli *et al.*, 1987). This unusual structure differed from the single hairpin-loop found in other arenavirus S RNAs but was confirmed by sequence analyses of several independently generated cDNA clones (Ghiringhelli *et al.*, 1991).

A structure identical to the one described for MC2 S RNA has now been determined in the vaccine strain Candid #1. It is interesting to note that,

a new corrected version of the Tacaribe virus S RNA, that was made available to us (M.T. Franze-Fernández, personal communication), also showed two potential hairpin-loop structures in the intergenic region. Although, the nucleotide sequence that forms the double hairpin-loop in Tacaribe S RNA is quite similar to the homologous stretch in Junín virus, the complete intergenic region is much longer.

More recently, Wilson and Clegg (1991) published the S RNA sequence of the African arenavirus Mopeia and showed two sets of self-complementary sequences capable of forming two hairpin loops separated by 24 non-base-paired nucleotides.

Final comments

Significant progress has been made in understanding the genetic basis of *attenuation* of the type 1 poliovirus vaccine strain using the recombinant viruses obtained by *in vitro* manipulation of infectious cDNA (Nomoto *et al.*, 1987; Racaniello and Baltimore, 1981). This approach has proved to be very productive because the changes at the nucleotide level can be precisely directed and the resulting phenotypes analyzed for changes in the biological properties. Unfortunately, at this time, it is quite difficult to regenerate infectious viruses from cDNA of segmented RNA genomes (Luytjes et al., 1989). Therefore, we had to resort to the more classical approach of analyzing the genomes of spontaneously generated mutants with different virulence phenotypes. To this end, we undertook the analysis of the variants derived in the process of development of the Candid #1 strain.

The attenuated Junin virus strains Candid #1 and XJ#44 analyzed in this report were compared to a wild type virus with no known genealogic relationship to the XJ lineage (Fig. 1). Nevertheless, the fact that the

less attenuated XJ#44 strain contains some structural features that are analogous to the virulent MC2 strain, and others that show its relatedness to the most attenuated Candid #1, is suggestive of the biological significance of the experimental data presented here. The molecular analyses of the more virulent predecessors of XJ#44 and Candid #1 (e.i., XJ#13 and XJ-Parodi) are underway and should shed more light on the relevance of the changes found at the genome level.

On the other hand, the virulence of a particular strain or virus passage should be regarded as the result of a complex population containing subpopulations of virions that contribute to the net pfu/LD50 ratio. This might be more important for uncloned RNA viruses like Junín-XJ#44 virus.

The RT-PCR assay and the simplified sample preparation procedure developed in our laboratory will allow a more thorough analysis of the variability of arenaviruses and the correlation of particular genotypic changes with the biological properties of viruses, such as disease patterns and virulence (Lozano *et al*, 1992; McKee *et al*, 1985 and 1987).

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Table 1. Sequence identity[a] of Junin virus strains and Tacaribe virus structural proteins

				GPC					
	N			G1			G2		
Virus[b]	XJ44	CD1	TAC	XJ44	CD1	TAC	XJ44	CD1	TAC
MC2	98.2	98.2	76.5	90.1	90.7	48.2	99.2	99.2	81.0
XJ44		99.6	78.1		99.4	54.8		98.3	81.0
CD1			78.1			53.4			80.6

[a]The amino acid sequences of all the proteins were aligned and compared pairwise. The figures indicate the percentage of identical amino acid residues in identical positions.
[b]MC2, Junin virus MC2 strain; XJ44, Junin virus XJ44 strain; CD1, Junin virus CD1 strain; TAC, Tacaribe virus

Table 2. Sequence homology[a] of three Junin virus strains and Tacaribe virus structural proteins

				GPC					
	N			G1			G2		
Virus[b]	XJ44	CD1	TAC	XJ44	CD1	TAC	XJ44	CD1	TAC
MC2	98.9	98.9	87.8	92.1	92.4	68.7	99.2	99.6	91.6
XJ44		99.6	88.6		99.7	72.5		98.7	90.7
CD1			88.4			72.7			91.1

[a]The amino acid sequences of all the proteins were aligned and compared pairwise. The figures indicate the percentage of similar amino acid residues including conservative changes, according to Schwartz & Dayhoff (1979), in identical positions.
[b]MC2, Junin virus MC2 strain; XJ44, Junin virus XJ44 strain; CD1, Junin virus CD1 strain; TAC, Tacaribe virus

TABLE 3

Localization of nucleotide and amino acid changes in the glycoprotein genes of wild type MC2 and vaccine Candid#1 strains of Junin virus

position		nucleotide	codon	amino acid
MC2	CD1	MC2 → CD1	MC2 → CD1	MC2 → CD1
--	1	- C	upstream	untranslated
37	38	A → G	non coding	region
214	*216	ins. U	stepwise	
218	*221	ins. C	change of	
225	*229	ins. U	reading	
226	231	ins. U	frames	CSILD → LFQFFVF[a]
228	*234	ins. U	(2 codons	
229	*236	ins. C	extra)	
234	242	6 nt. block	--- CUC	
to	to	insertion		-- → LA[b]
235	247	CUCGCA	--- GCA	
247	260	C → A	stepwise	
248	262	ins. A	change of	
250	265	ins. A	reading	PR → TEE[c]
252	268	ins. A	frames	
			(1 codon extra)	
280	295	del. G	stepwise	
300	314	del. U	change of	VPDCVLLQWWVS → FQTVSFSMVGL[d]
315	*333	del. U	reading	
			frames	
			(1 codon loss)	
502	515	A → U	AGG → UGG	R → W[e]
1123	1136	U → C	UUG → CUG	L → L
1239	1252	U → C	AUU → AUC	I → I
1264	1277	C → U	CUA → UUA	L → L
1323	1336	C → U	GGC → GGU	G → G
1332	1345	C → U	CCC → CCU	P → P
1354	1367	U → A	UUU → AUU	F → I[f]
1383	1396	C → A	UCC → UCA	S → S
1404	1417	C → U	GGC → GGU	G → G
1411	1424	A → U	ACC → UCC	T → S[g]
1416	1429	U → C	CAU → CAC	H → H
1419	1432	G → A	AGG → AGA	R → R
1428	1441	A → G	CAA → CAG	R → R
1434	1447	G → A	CGG → CGA	E → E
1444	1457	C → U	CUG → UUG	L → L
1449	1462	C → A	CCC → CCA	P → P
1456	1469	C → U	CUA → UUG	L → L
1458	1471	A → G	CUA → UUG	L → L
1461	1474	U → C	AAU → AAC	N → N
1521	1534	C → U	CGC → CGU	R → R
1541	1554	C → U	downstream	untranslated
1544	1557	A → G	non coding	region
1565	1578	U → C		

* In insertions or deletions in which a stretch of the same nucleotide is involved, it is not possible to specify the exact position of the mutational change. Therefore, the last nucleotide in the stretch is indicated to identify the sequence position.

a,b,c,d,e,f,g: refer to the regions indicated in Fig.

Candid #1 genealogy

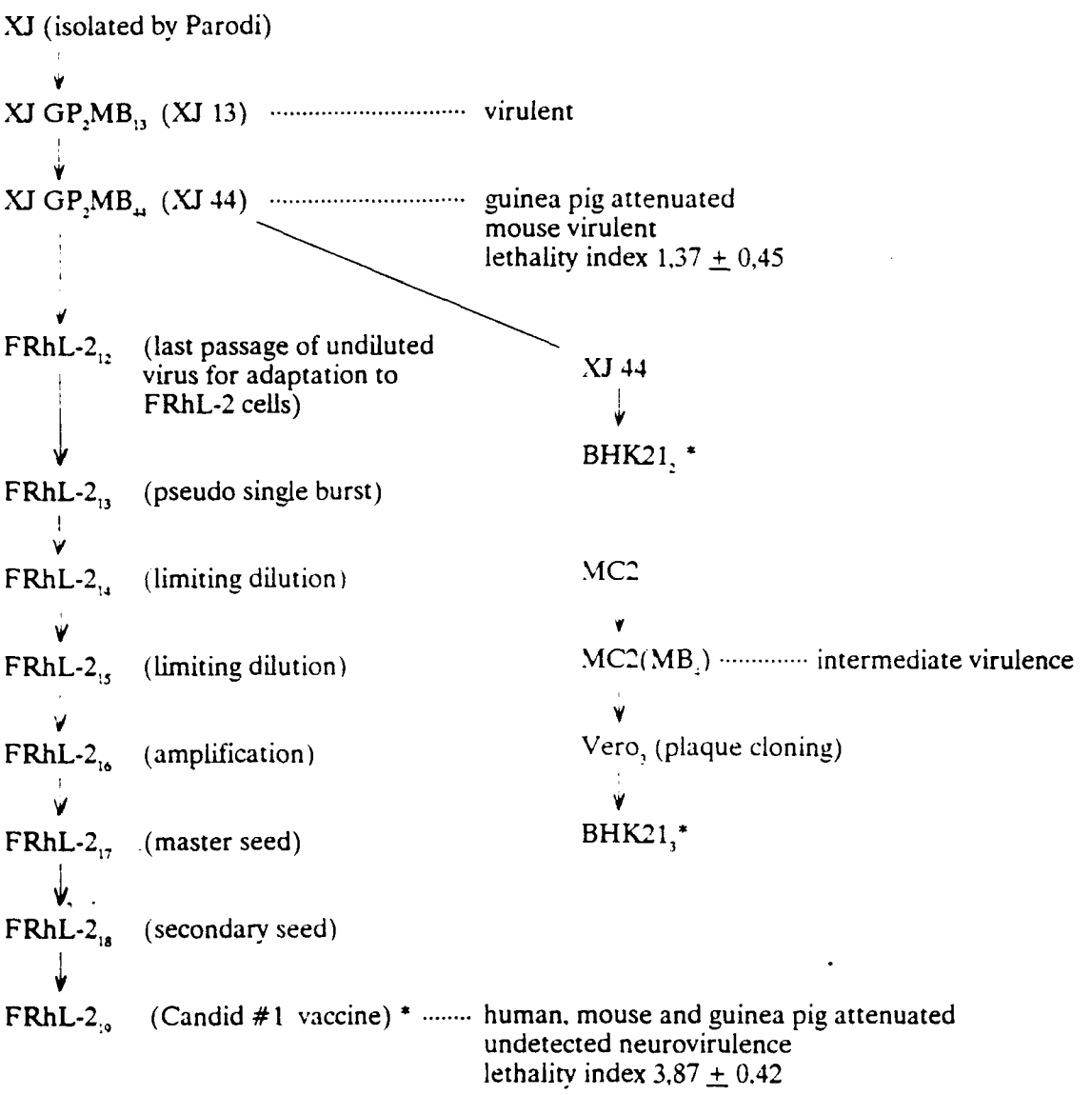

Fig. 1 Passage history of Junin virus strains. The records of the passages of the XJ prototype Junin virus isolated by Parodi come from the Yale Arbovirus Research Unit (Dr. J. Casals) and USAMRIID (Dr. J.G. Barrera-Oro). GP_n stands for n passages in guinea pig and MB_n for passages in mouse brain (for simplicity, the ages of the experimental animals are not indicated). The attenuated Junin virus XJ44 was passaged in Fetal Rhesus Lung diploid cells (FRhL-2) to generate the vaccine virus Candid #1. The MC2 strain is an independent isolate and was propagated in suckling mouse brain, plaque-cloned in Vero cells and grown in BHK-21 cells. The asterisks (*) indicate the passage level of the virions used as the source for RNA isolation and molecular cloning.

The lethality index was calculated as the $\log_{10}$ pfu that produce one LD_{50} (± standard deviation) by intracerebral route in mice.

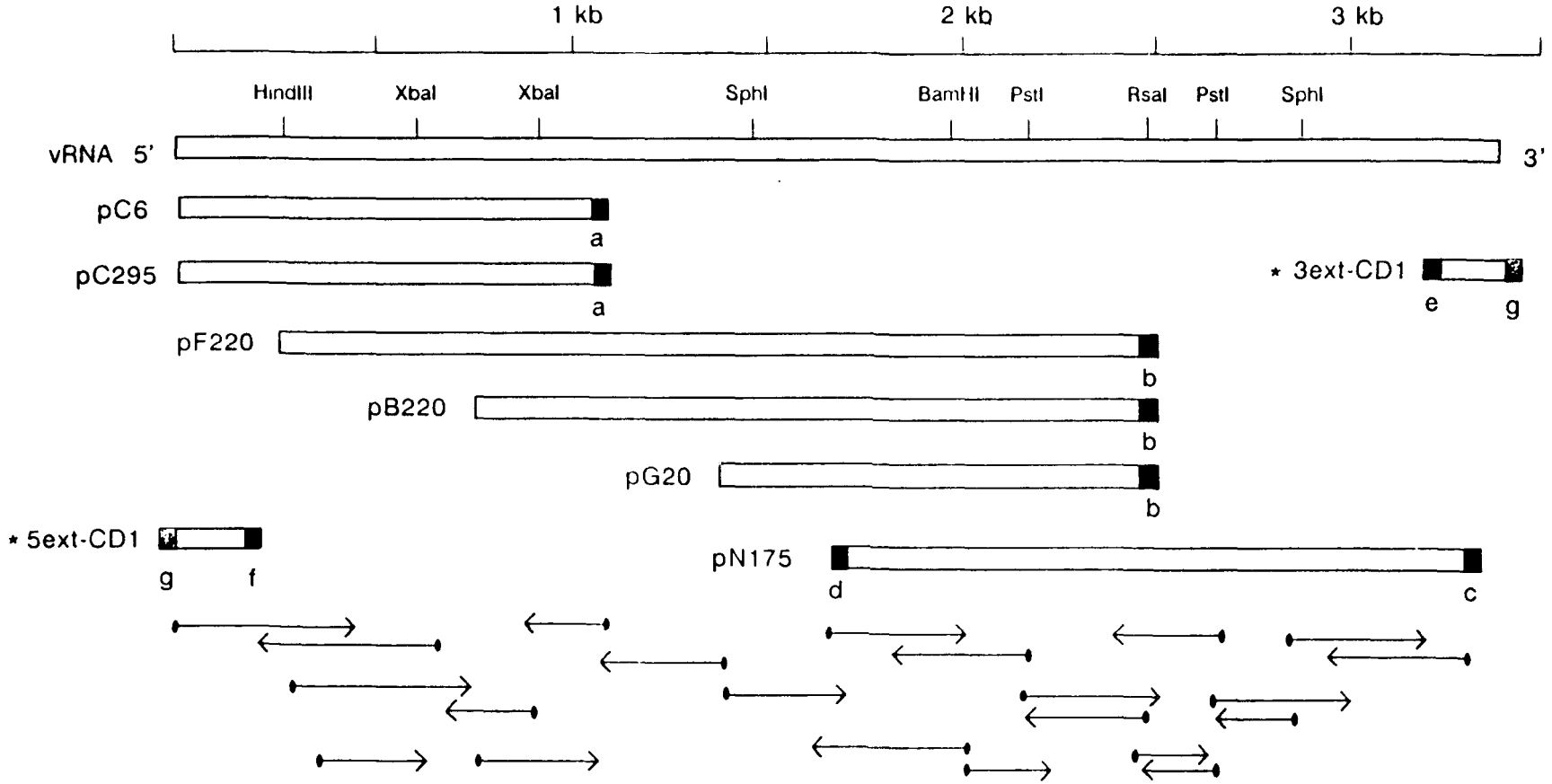

Figure 2. Cloning and sequencing strategy of Junin virus S RNA (Candid 1 strain) . The S RNA nucleotide sequence is represented in the viral sense. The overlapping clones, obtained by cDNA synthesis and cloning or by RT-PCR and cloning span the entire S RNA sequence (open rectangles). Synthetic oligonucleotides used to prime cDNA synthesis and for PCR amplification are shown as hatched or black boxes at the ends of each clone , and designated with lower case letters (a-g) . The oligonucleotides were designed according to the MC2 strain S RNA sequence (Ghiringhelli *et al.*, 1991) . Hatched boxes indicate the oligo-dG primer used to amplify the primer extension products tailed with oligo-dC (see materials and methods) . Subcloning of the DNA fragments into M13mp18 and mp19 , using the restriction sites indicated, was performed to generate nucleotide sequence data. The sequences were obtained using *Sequenase* ™ as shown by the arrows (Sanger *et al* , 1977) . The clones marked with asterisks have not been sequenced yet.

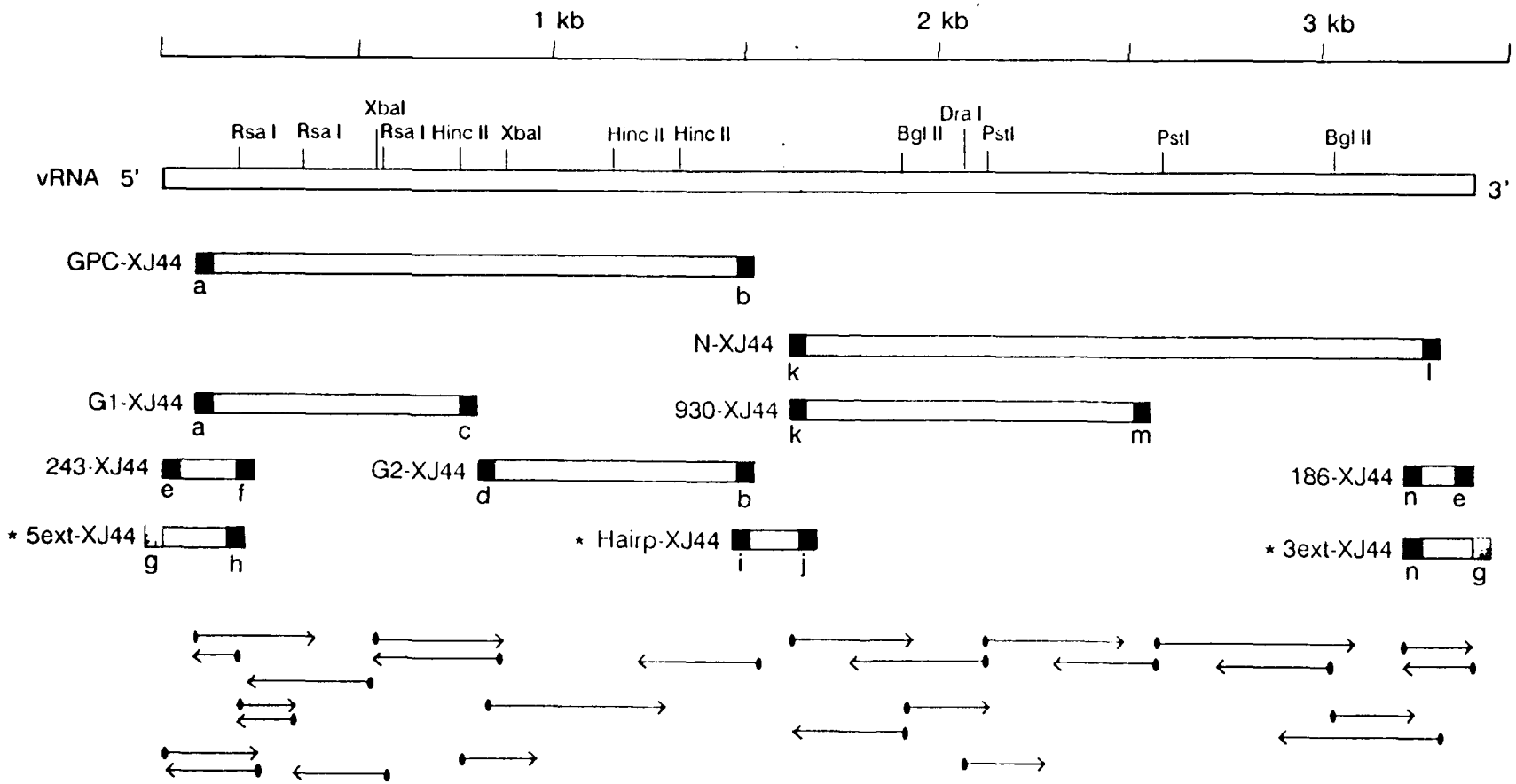

Figure 3. Cloning and sequencing strategy of Junin virus S RNA (XJ 44 strain) . The S RNA nucleotide sequence is represented in the viral sense. The overlapping clones, obtained by PCR, spanning the entire S RNA, are shown as open rectangles. Synthetic oligonucleotides used to prime cDNA synthesis and for PCR amplification are shown as hatched or black boxes at the ends of each clone, and designated with lower case letters (a-n) . The oligonucleotides were designed according to the MC2 strain S RNA sequence (Ghiringhelli *et al.*, 1991) . Hatched boxes indicate the oligo-dG primer used to amplify the primer extension products tailed with oligo-dC (see materials and methods).

Subcloning of the PCR fragments into M13mp18 and mp19 , using the restriction sites indicated, was performed to generate nucleotide sequence data. The sequences were obtained using *Sequenase* ™ as shown by the arrows (Sanger *et al.*, 1977). The clones marked with asterisks have not been sequenced yet.

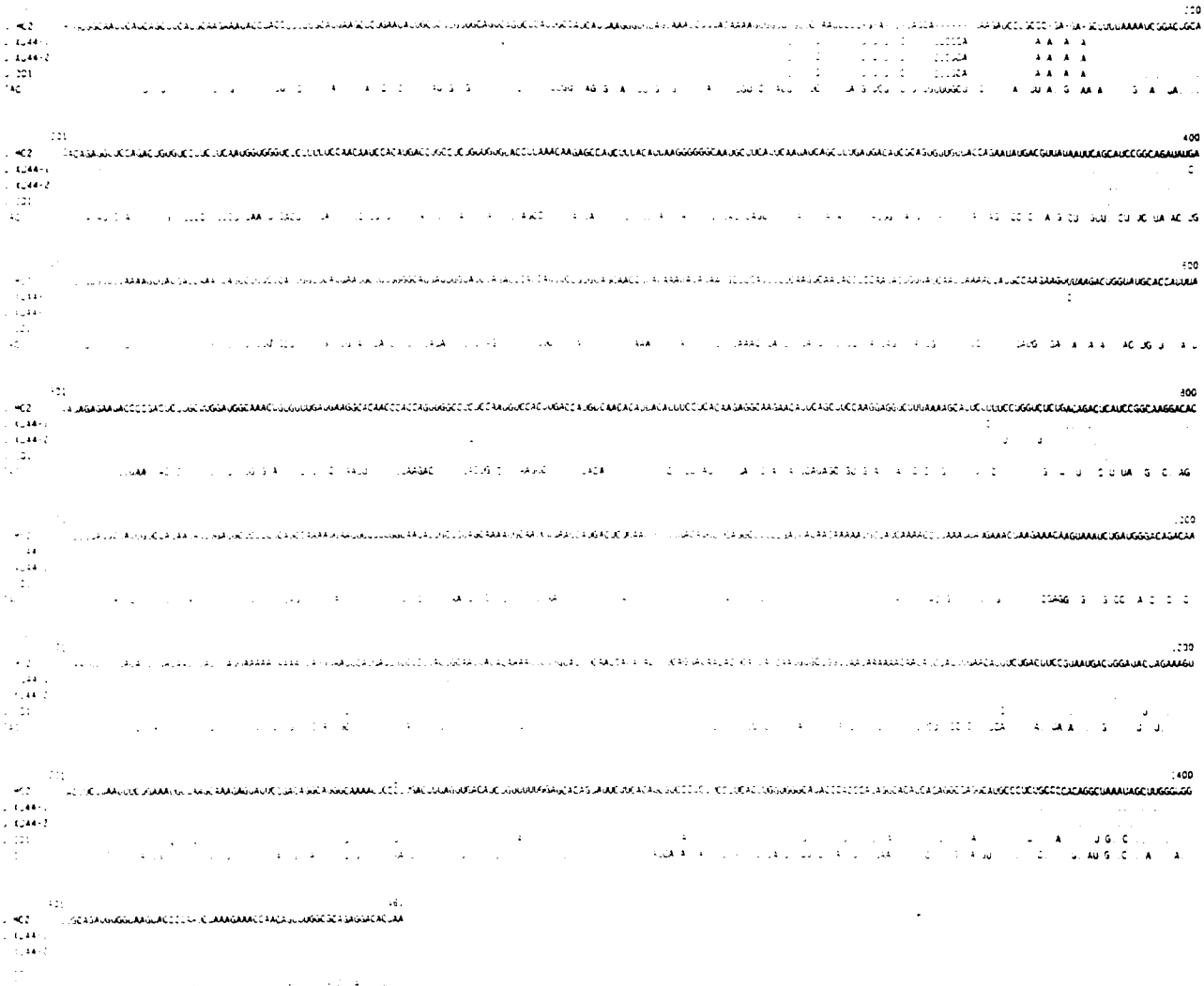

Figure 4. Nucleotide sequence comparison of the GPC genes of three strains of Junin virus. The viral sense RNA sequences of the Junin virus strains MC2 (J.MC2; Ghiringhelli *et al.*, 1991), XJ44 (two independent cDNA clones J.XJ44-1 an J.XJ44-2) and Candid #1 (J.CD1) were aligned starting at the AUG initiation codon of GPC and ending at the UAA stop codon. The Tacaribe arenavirus (TAC; Franze-Fernandez *et al.*, 1987) GPC ORF was included in the comparison for its close serologic relationship with Junin virus. The nucleotide numbering in this figure corresponds to the GPC ORF and includes the gaps (hyphens) necessary for the alignment. The published Junin MC2 nucleotide sequence is used as reference and the homologous nucleotides in the other viral sequences are indicated by dots; only the nucleotide changes are shown.

```
  1   MGQFISFMQEIPTFLQEALNIALVAVSLIAIIKGVVNLYKSGLFQFFVFLALAGRSCTEE
                                                -CSILD-  --    PR-

 61   AFKIGLHTEFQTVSFSMVGLFSNNPHDLPLLCTLNKSHLYIKGGNASFKISFDDIAVLLP
            VPDCVLLQWWVS

121   EYDVIIQHPADMSWCSKSDDQIWLSQWFMNAVGHDWYLDPPFLCRNRTKTEGFIFQVNTS
                              R

181   KTGINENYAKKFKTGMHHLYREYPDSCLDGKLCLMKAQPTSWPLQCPLDHVNTLHFLTRG

241   KNIQLPRRSLKAFFSWSLTDSSGKDTPGGYCLEEWMLVAAKMKCFGNTAVAKCNLNHDSE
          • •

301   FCDMLRLFDYNKNAIKTLNDETKKQVNLMGQTINALISDNLLMKNKIRELMSVPYCNYTK

361   FWYVNHTLSGQHSLPRCWLIKNNSYLNISDFRNDWILESDFLISEMLSKEYSDRQGKTPL

421   TLVDICIWSTVFFTASLFLHLVGIPSHRHIRGEACPLPHRLNSLGGCRCGKYPNLKKPTV
            F                     T

481   WRRGH
```

Figure 5. Amino acid sequence of Junin virus Candid #1 GPC gene products. The regions were Candid #1 GPC sequence differs from that of the wt MC2 strain are identified by letters a through g (for easy cross-reference with table 3) and underlined. The amino acid sequence of the MC2 GPC protein in these regions appears below the lines. The putative N-glycosilation sites are boxed. Two dots (• •) highlight the double basic amino acid sequence identified by Buchmeier *et al.* (1987) as the putative proteolityc cleavage site that generates G1 (NH_2-terminal half) and G2 (COOH-terminal half of GPC)

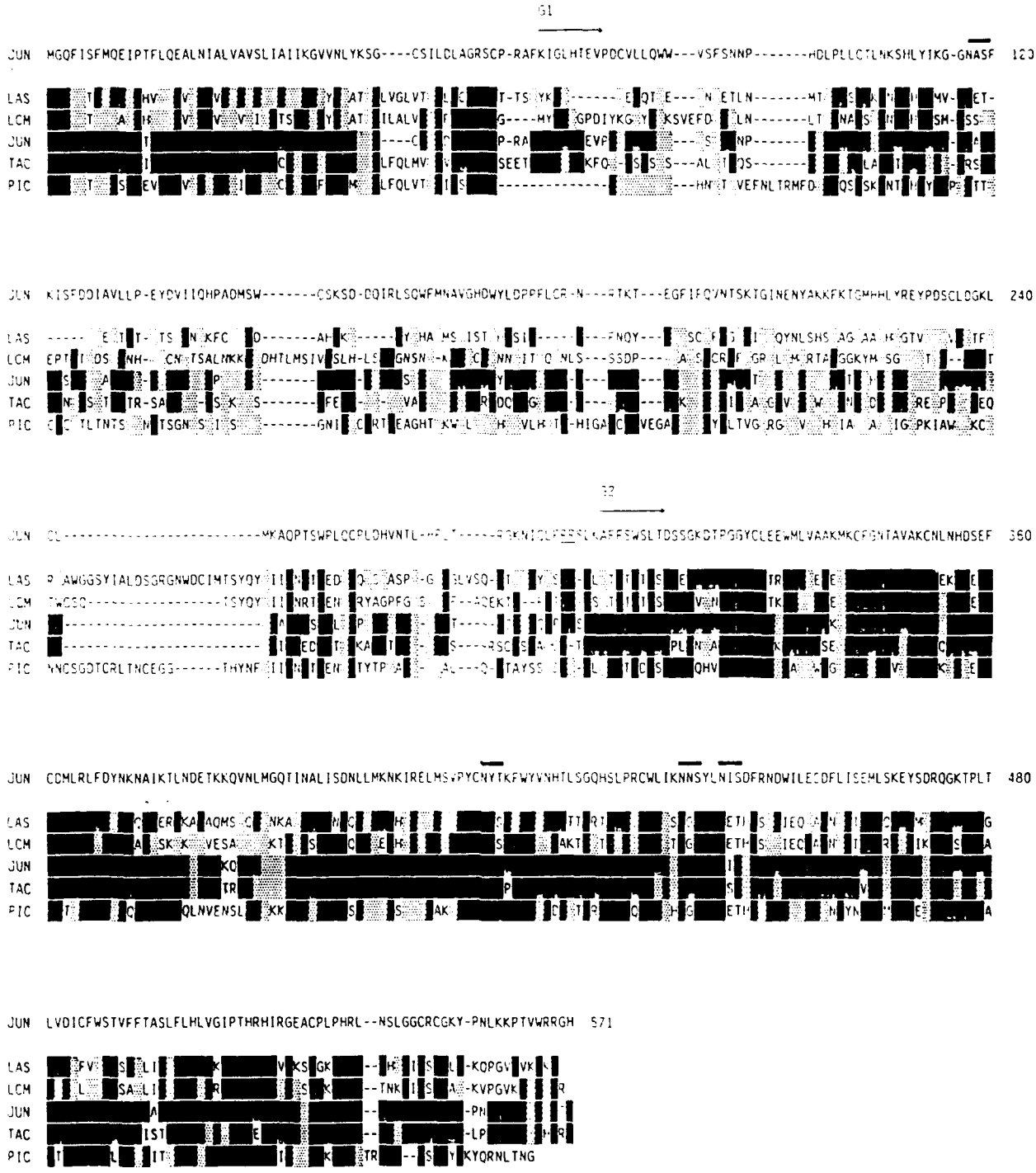

FIGURE 6: Comparison of arenavirus GPC proteins

The predicted amino acid sequences of the GPC proteins of LAS (Auperin *et al.*, 1986), LCM (Romanowski *et al.*, 1985), TAC (Franze-Fernandez *et al.*, 1987) and PIC (Auperin *et al.*, 1984) are compared to that of JUN GPC. The amino acid residues identical to those of JUN N protein are indicated by black boxes; those that represent conservative changes according to Schwartz and Dayhoff (1979) appear as shaded areas. Gaps were incorporated in the sequences in order to obtain a best fit alignment. The reference JUN GPC sequence is printed above the aligned sequences. In addition, the conserved N-glycosylation sites (NX_S^T) are indicated by thick lines above the JUN GPC sequence. The approximate position of the proteolytic cleavage site is underlined (the amino acid sequence to the left of this site is referred as G1 and that, to the right, as G2). The arrows start at the amino termini of G1 and G2 of LCM sequenced by Burns *et al.* (1990).

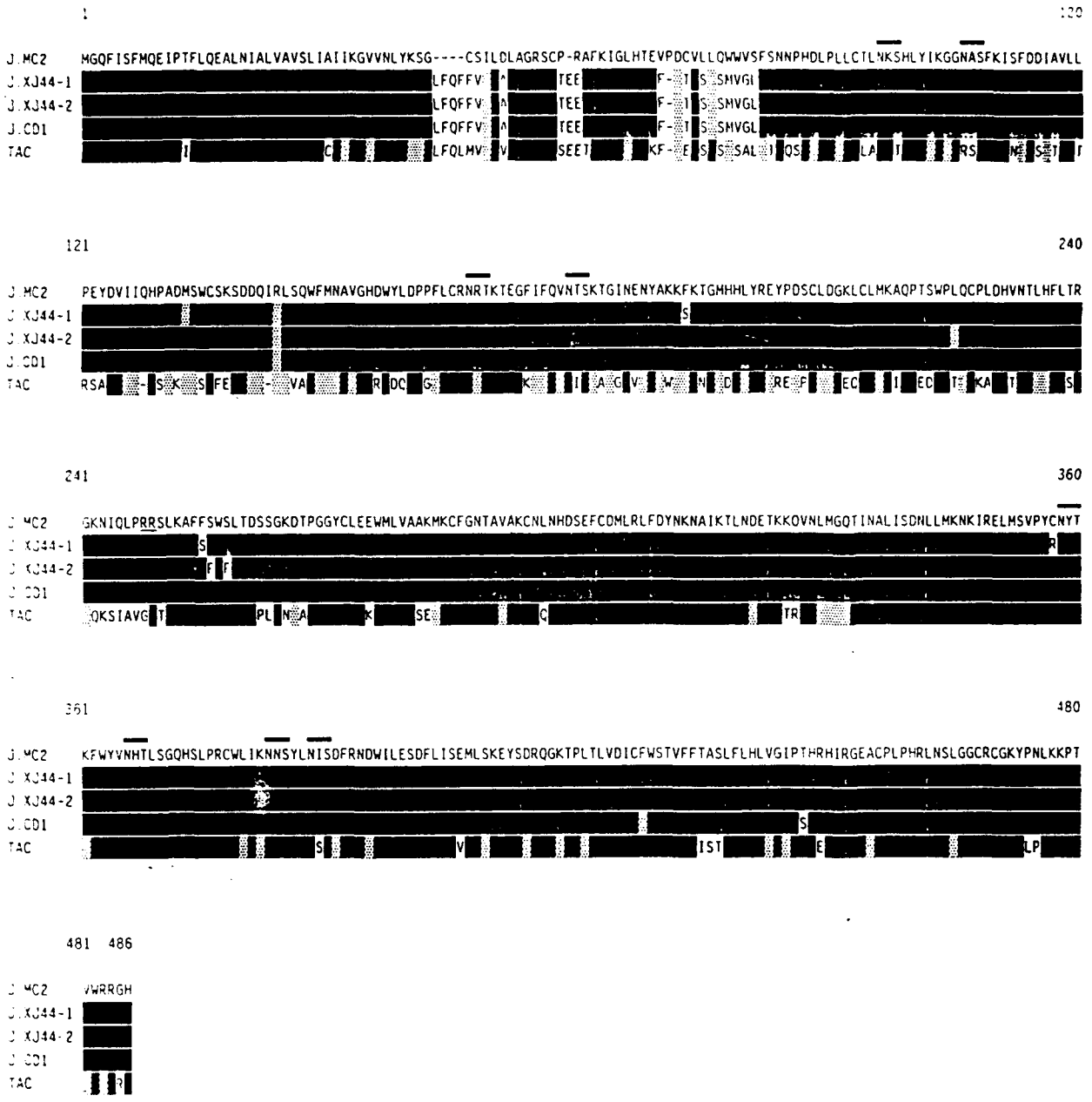

Figure 7: Comparison of the GPC protein of three strains of Junin virus. The amino acid sequences of the Junin virus strains MC2 (J.MC2; Ghiringhelli *et al.*, 1991), XJ44 (two independent cDNA clones J.XJ44-1 and J.XJ44-2) and Candid #1 (J.CD1) was compared. The Tacaribe arenavirus (TAC; Franze-Fernandez *et al.*, 1987) GPC was included in the comparison for its close serologic relationship with Junin virus. The identities and homologies of amino acid residues are indicated as in Figure 6.

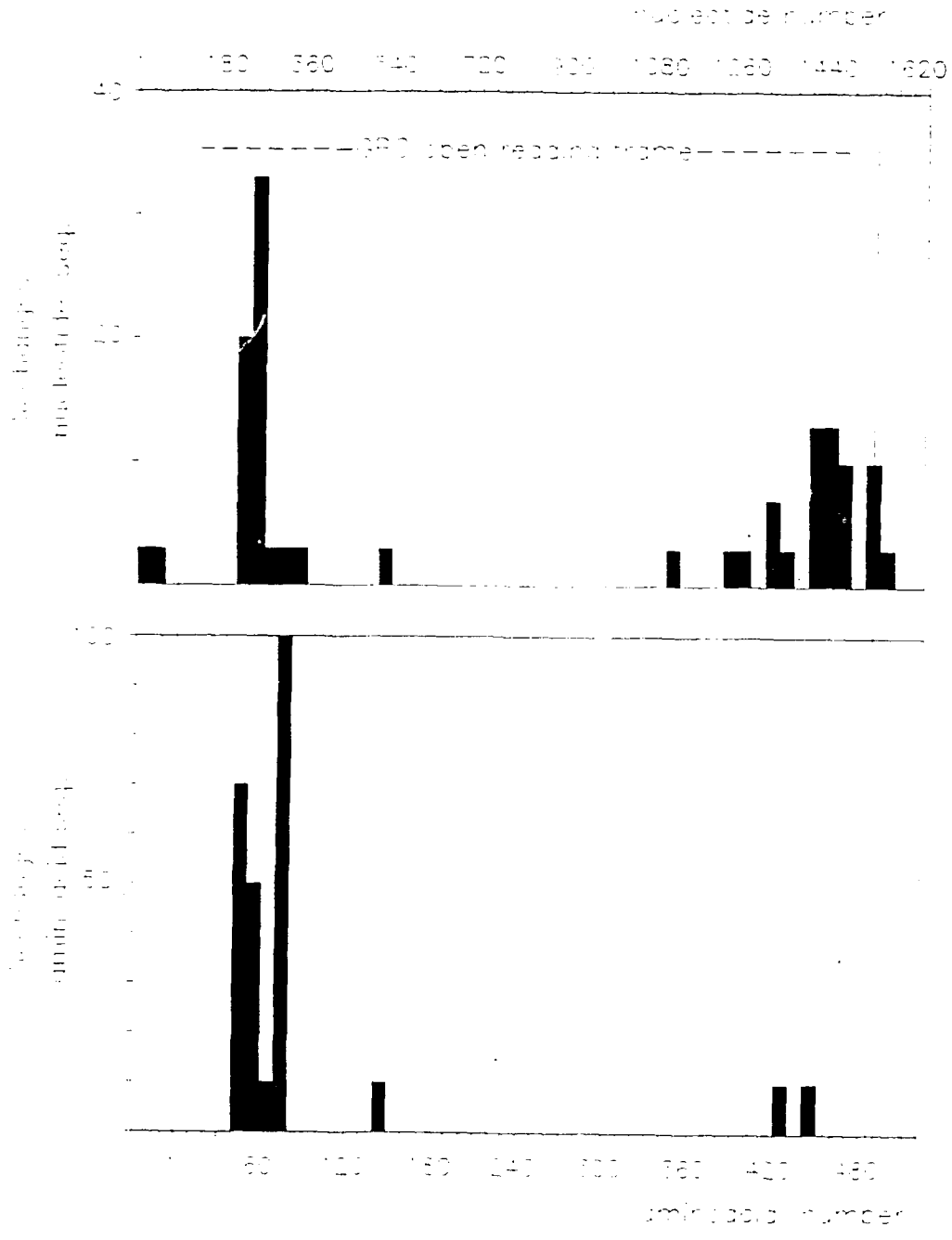

FIGURE 8: Distribution of the amino acid and nucleotide changes in the GPC protein, the corresponding open reading frame and the 5' and 3' untranslated regions. The nucleotide and amino acid sequences of Candid #1 and MC2 strains, aligned as in Fig. 4 and 6, were subdivided in fractions of 30 nucleotides and 10 amino acids, respectively, in order to score the differences found while scanning the sequences. The upper panel is a histogram-plot of the nucleotide changes (%) calculated from the pairwise comparison of the homologous 30-nucleotide fragments of Candid #1 and MC2 S RNAs. The boundaries of the GPC ORF are indicated by two vertical lines, that continue into the lower panel. In this panel, the changes (%) in the amino acid sequence of the 10-amino-acid-residue fractions of Candid #1 and MC2 GPC proteins were plotted.

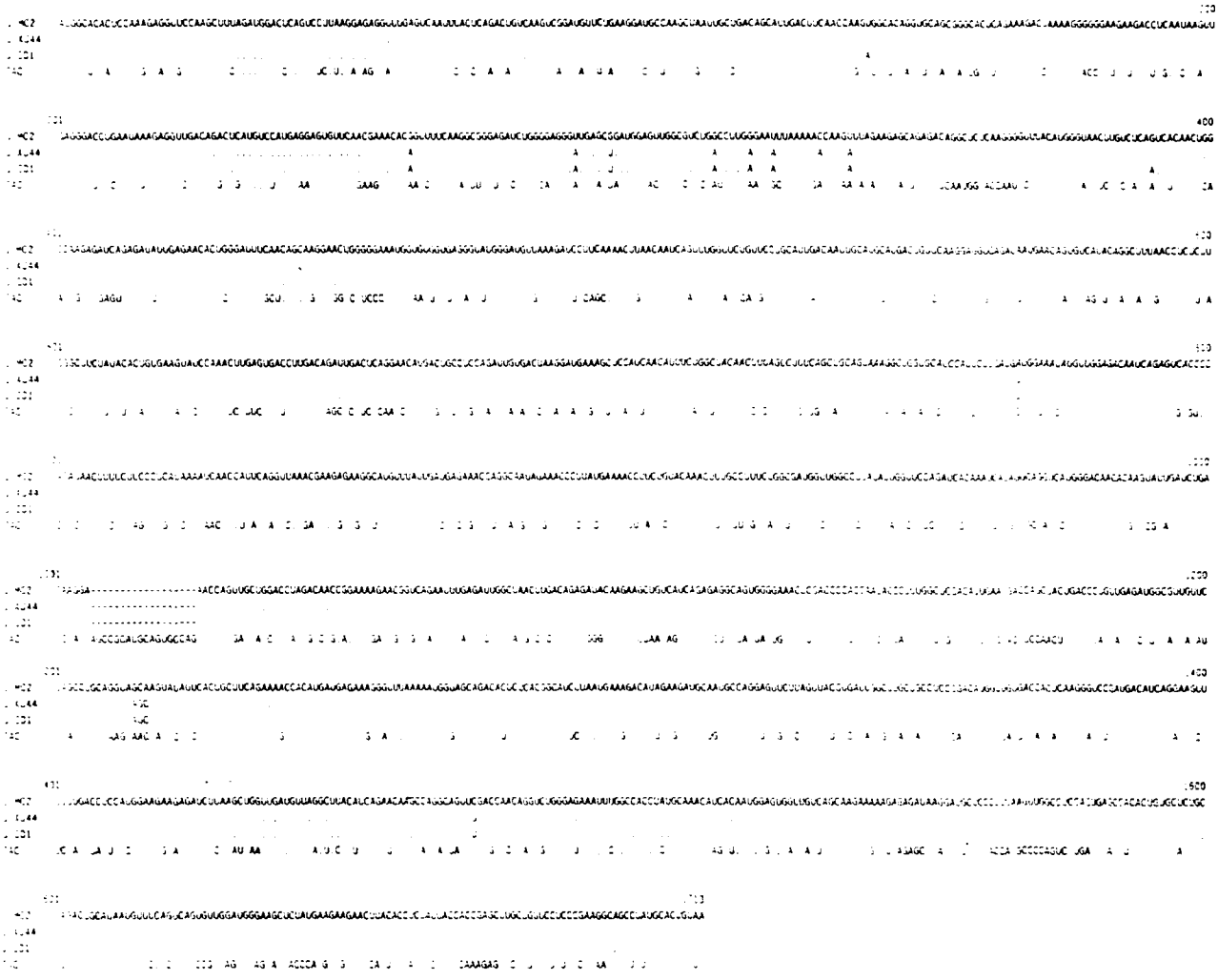

Figure 9. Nucleotide sequence comparison of the N genes of three strains of Junin virus. The viral complementary or message sense RNA sequences of the Junin virus strains MC2 (J.MC2; Ghiringhelli *et al.*, 1989), XJ44 (J.XJ44) and Candid #1 (J.CD1) were aligned starting at the AUG initiation codon of N and ending at the UAA stop codon. The Tacaribe arenavirus (TAC; Franze-Fernandez *et al.*, 1987) N ORF was included in the comparison for its close serologic relationship with Junin virus. The nucleotide numbering in this figure corresponds to the N ORF and includes the gaps (hyphens) necessary for the alignment. The published Junin MC2 nucleotide sequence is used as reference and the homologous nucleotides in the other viral complementary sequences are indicated by dots; only the nucleotide changes are shown.

MAHSKEVPSFRWTQSLRRGLSQFTQTVKSDVLKDAKLIADSIDFNQEAQVQRALRKTKRG
V

EEDLNKLRDLNKEVDRLMSMRSVQRNTVFKAGDLGRDELMELASDLEKLKTKIRRAETGS
V R G GN F

QGVYMGNLSQSQLAKRSEILRTLGFQQQGTGGNGVVRVWDVKDPSKLNNQFGSVPALTIA

CMTVQGGETMNSVIQALTSLGLLYTVKYPNLSDLDRLTQEHDCLQIVTKDESSINISGYN

FSLSAAVKAGASILDGGNMLETIRVTPDNFSSLIKSTIQVKRREGMFIDEKPGNRNPYEN
D

LLYKLCLSGDGWPYIGSRSQIIGRSWDNTSIDLTRKPVAGPRQPEKNGQNLRLANLTEIQ

EAVIREAVGKLDPTNTLWLDIEGPATDPVEMALFQPAGKQYIHCFRKPHDEKGFKNGSRH
SK

SHGILMKDIEDAMPGVLSYVIGLLPPDMVVTTQGSDDIRKLFDLHGRRDLKLVDVRLTSE

QARQFDQQVWEKFGHLCKHHNGVVVSKKKRDKDAPFKLASSEPHCALLDCIMFQSVLDGK

LYEEELTPLLPPSLLFLPKAAYAL

Figure 10: Amino acid sequence of Junin Candid #1 virus N protein.
Translation product of the N ORF. The clusters of two, three and four basic amino acid residues K and R are boxed. The amino acid residues that are different in the MC2 strain N protein are shown under the Candid #1 sequence.

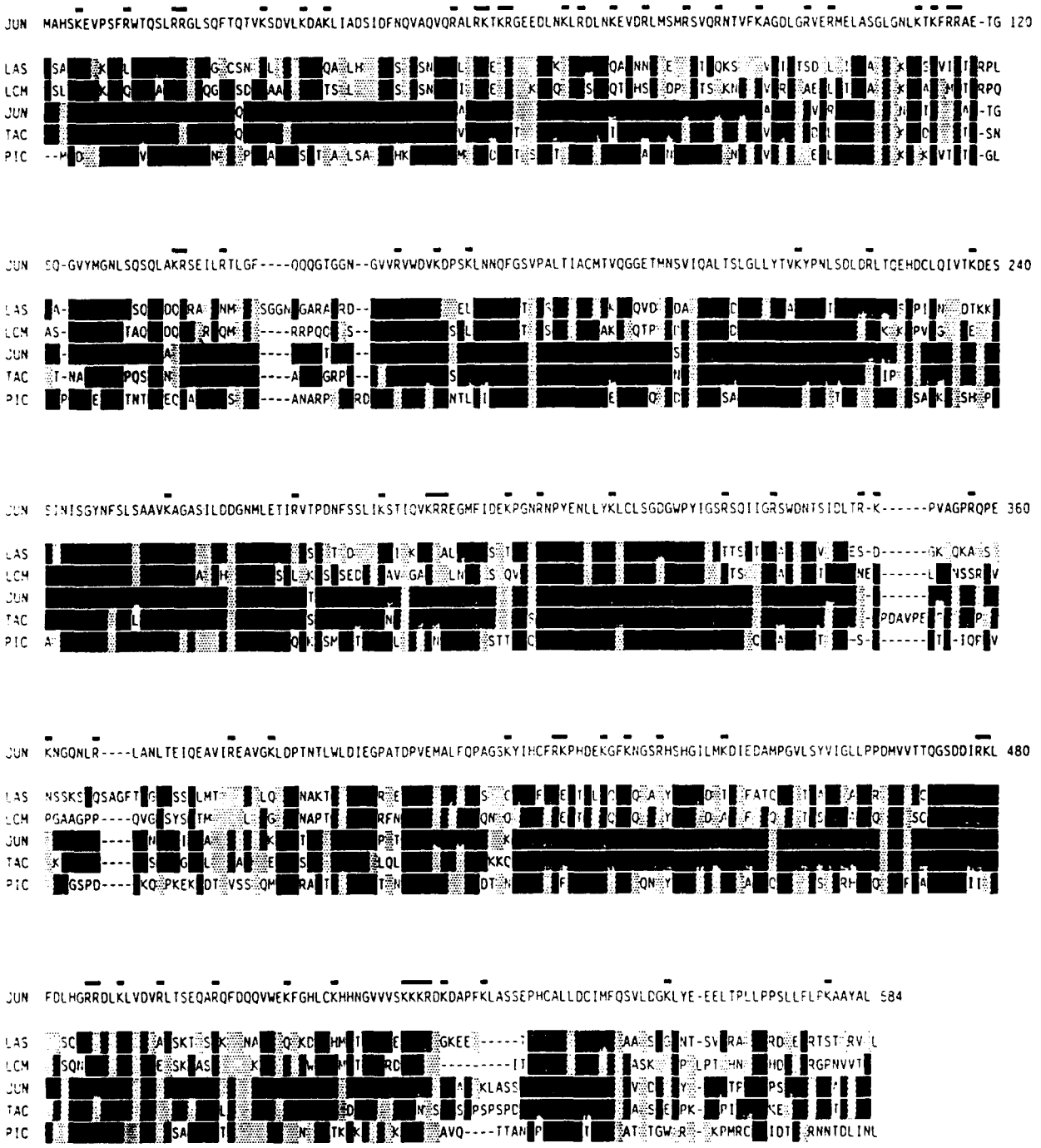

FIGURE 11: Comparison of the amino acid sequence of the N proteins of the Old World arenaviruses Lassa (LAS; Clegg and Oram, 1985) and LCM-WE (LCM; Romanowski and Bishop, 1985), and the New World arenaviruses Tacaribe (TAC; Franze-Fernandez *et al.*, 1987) and Pichinde (PIC; Auperin *et al.*, 1984) with that of Junin N polypeptide (JUN; Ghiringhelli *et al.*, 1989). The identities and homologies of amino acid residues are indicated as in Figure 6. The complete amino acid sequence of JUN N protein, that was used as reference, is printed above the comparison of the five N proteins (Ghiringhelli *et al.*, 1991). The basic amino acids residues K and R are highlighted with thick lines above the sequence of Junin virus N.

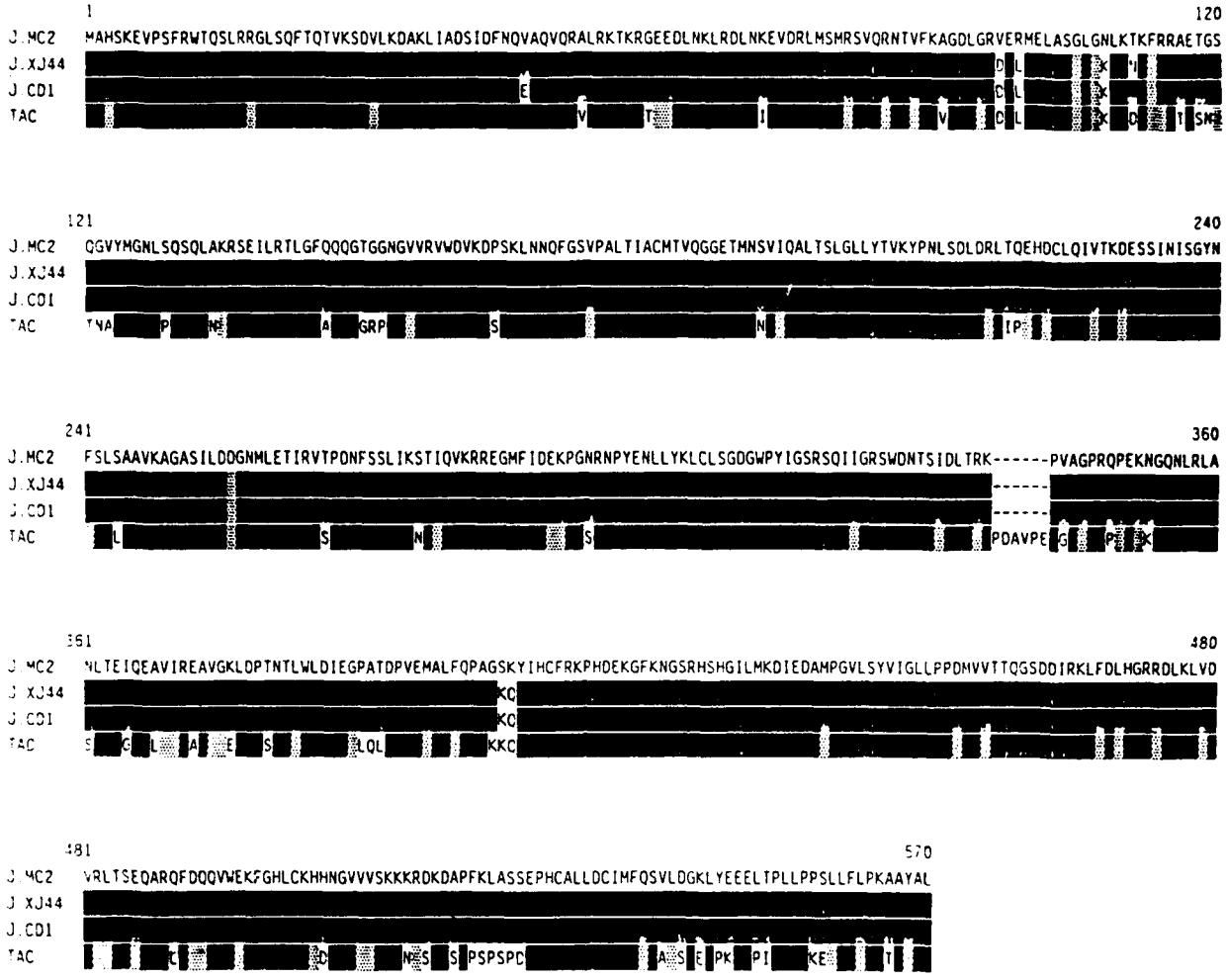

Figure 12: Comparison of the N protein of three strains of Junin virus. The amino acid sequences of the Junin virus strains MC2 (J.MC2; Ghiringhelli *et al.*, 1991), XJ44 (J.XJ44) and Candid #1 (J.CD1) was compared. The Tacaribe arenavirus (TAC; Franze-Fernandez *et al.*, 1987) N was included in the comparison for its close serologic relationship with Junin virus. The identities and homologies of amino acid residues are indicated as in Figure 6.

A

```
                1                   22                                                                  93
J.MC2 D-58      UGCAGUAAGGGGAUCCUAGGCGA-UUUUGGUAACGCUAUAAGUUGUUACUGCUUUCUAUUUGGACAACAUCAAA-CCAUCUAUUGUACAAUG
J.MC2 B-201     ........................-.....................................................-.............
J.MC2 1-8       ..-.......U.........-....A....U.....G.................AA....C....C......
J.MC2 1-10      ..-.......U.........-....A....U.....G.................AA....C....C......
J.MC2 1-13      ..-.......U.........-....A....U.....G.................AA....C....C......
J.MC2 2-48      ..-.......U.............A....U.....G.................-.....C....C......
J.MC2 2-52      ..U.......U.............A....U.....G.................-.....C....C......
J.MC2 2-59      ..-.......U.............A....U.....G.................-.....C....C......
J.MC2 2-150     ..-.......U.........-....A....U.....G.................AA....C....C......
J.XJ44-70       ..-.......U.........-....A....U.....G.................AA....C....C......
J.XJ44-61       ..-.....................-....A....U.....G.................---....C....C......
J.CD1 6         ........................-.............G.....................................-.............
J.CD1 295       ........................-.............G.....................................-.............
TAC             C...CC--.............-.U..C.J..CCAUA.U.GCC.------------------A..UGA.C..GGUGA...AC.CCC..C...
```

B

```
                1                   20                                                                  86
J.MC2           CGCACAGUGGAUCCUAGGCAUAGUGUCUAACACUUUCACUGAGCAAUUUUUUUCUCAGAUCAAAACCACCAGAUCUUCUGGCAUG
J.XJ44                              ..................................................................
TAC             CGCACAGUGGAUCCUAGGC.A.U........U............UC.......----..AAUCCUUGCUUU....-G.CAUA...
```

Figure 13. Untranslated regions of Junin virus S RNA.

A: The nucleotide sequences of independent cDNA clones comprising the 5' end of the S RNA and the AUG codon that initiates the GPC ORF were aligned. The homologous bases are replaced by dots and the gaps introduced for a best fit alignment, by hyphens. The virus and strain identifications are the same as in figures 4 and 9 and are followed by the clone number. The sequences that start at nucleotide 22 were obtained from clones derived from PCR products and do not include the sequence of the primer used for the amplification (hybridizing to nucleotides 1 to 21).

B: Alignment of cDNA sequences complementary to the 3' end of the S RNA of Junin virus (strains MC2 and XJ44) and Tacaribe virus. The sequence of the XJ44 clone (J.XJ44) was obtained by RT-PCR amplification. The first 19 nucleotides are not shown because they correspond to the primer used in the PCR.

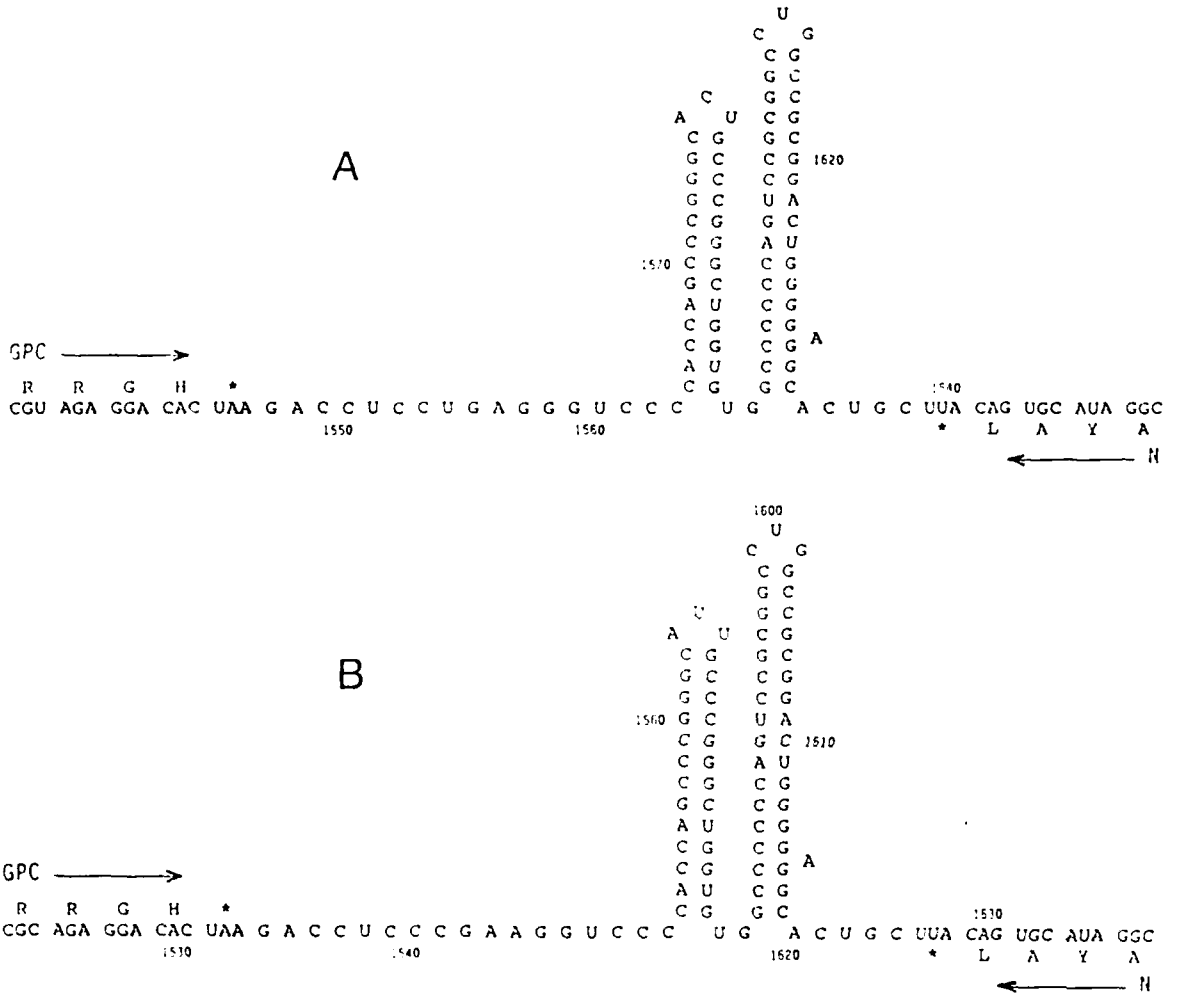

Figure 14: Intergenic region of Junin virus S RNA. A portion of the S RNA of Junin-Candid #1 virus comprising the ends of the GPC and N genes (sense and anti-sense strand, respectively) and the non-coding intergenic region is shown in the figure. The directions of translation are indicated by arrows and the stop codon for the GPC protein and the stop anticodon for the N gene are marked with asterisks. Two potential hairpin-loop structures are stabilized by 13 and 17 base pairs, respectively (ΔG^{o} = -39.0 and -54.2 kcal/ mole). In the lower part of this figure, the homologous region of Junin-MC2 virus S RNA is shown for comparison. The nucleotides C_{1541}, A_{1544} and U_{1565} of MC2 have been changed to U_{1554}, G_{1557} and C_{1578} in Candid #1.

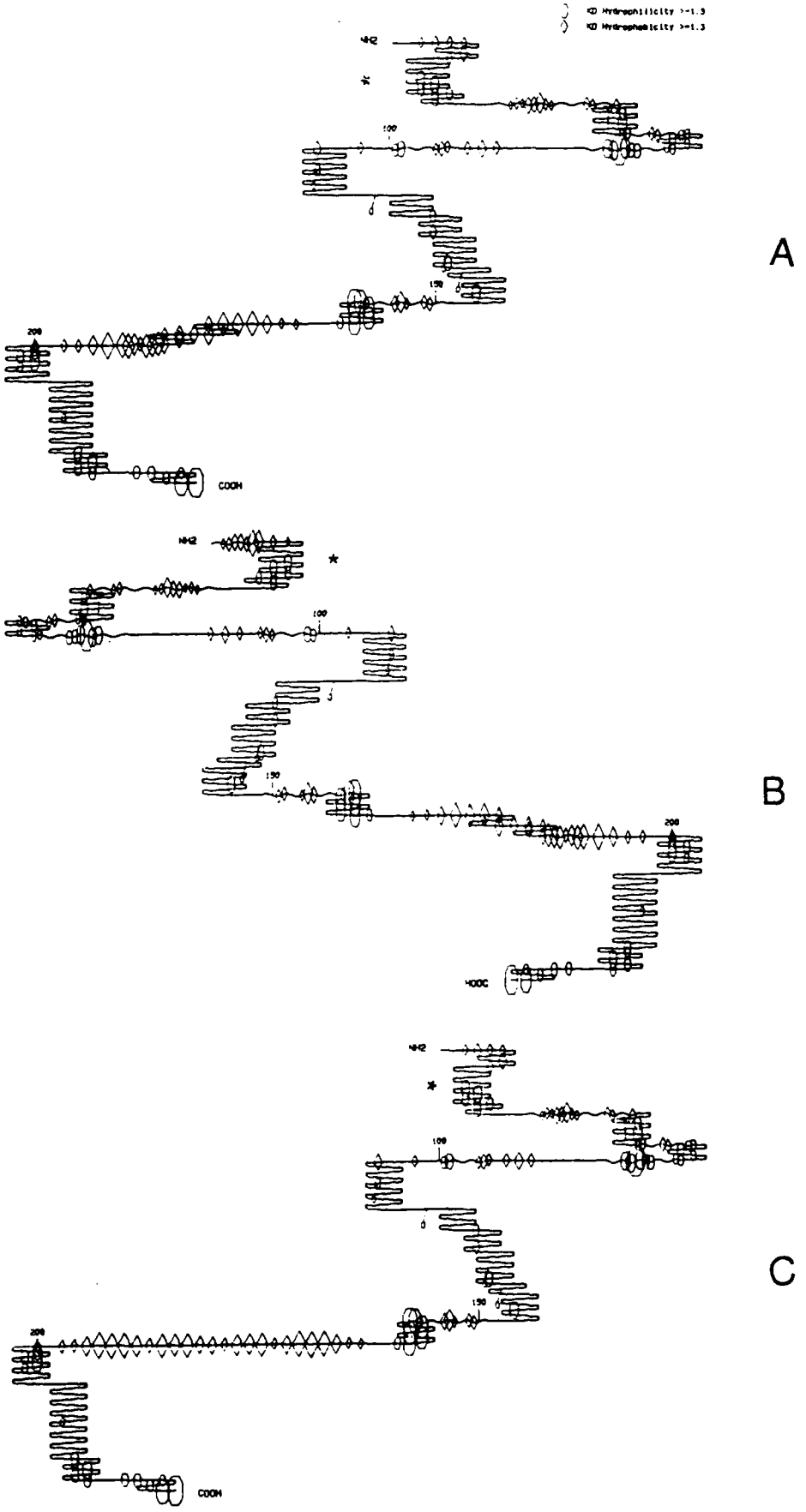

Figure 15: Predicted secondary structures and hydrophilicity of G2 proteins.
The secondary structure of G2 was predicted by the Garnier-Osguthorpe-Robson algorithm (a slight modification of Garnier et al., 1978; included in the Sequence Analysis Software by Genetics Computer, Inc., GCG Version 7.0). The diamonds indicate hydrophobic regions and the octagons, hydrophilic amino acid sequences. The plot presents the G2 regions of the GPC of the MC2 (A), the XJ#44 (B) and the Candid #1 (C) strains of JunIn virus. The change in the orientation of the XJ#44 G2 (B) in this plot is due to one B-turn less than those found in the other strains (A,C) in the region indicated by asterisks. Note the different secondary structures in the region of residues 173-198.

PUBLICATIONS AND MEETING ABSTRACTS

1. "**The glycoprotein precursor gene of the Junín virus vaccine strain**"
   V. Romanowski, P.D. Ghiringhelli, C.G. Albariño and M. Piboul
   10th Annual Meeting of the American Society for Virology, Colorado State University, Fort Collins (USA) 7-11 July, 1991.
   This paper presents the results and conclusions described in the midterm report.

2. "**A simple nucleic acids amplification assay for the early and rapid detection of Junín virus in clinical specimens.**"
   V. Romanowski, M.E. Lozano, P.D. Ghiringhelli and O. Grau
   8th International Conference on Negative Strand Viruses, Charleston, South Carolina (USA) 15-20 September, 1991.
   This paper describes a simplified procedure that rapidly inactivates nucleases and viruses and permits a successful enzymatic amplification of selected Junin virus S RNA sequences from tissue culture or whole blood specimens. These results are related to the subject of the grant, since the methodology might be used for the genotypic control of vaccine stocks, the analysis of AHF cases presumed to be vaccine-related, the more extensive studies of virus isolates with different phenotypes, etc.

3. "**Detección temprana de virus Junín en muestras clínicas por amplificación de ácidos nucleicos**"
   M.E. Lozano, P.D. Ghiringhelli, V. Romanowski y O. Grau
   XXVII Reunión Anual de la Sociedad Argentina de Investigación Bioquímica, Huerta Grande (Córdoba) 21-24 October, 1991.
   This is essentially the paper #2, presented at the Annual Meeting of the Argentine Society for Biochemical Research.

4. "**El gen del precursor de las glicoproteínas de la cepa de la vacuna del virus Junín (Candid #1)**"
   P.D. Ghiringhelli, C.G. Albariño, Mariel Piboul y V. Romanowski
   XXVII Reunión Anual de la Sociedad Argentina de Investigación Bioquímica, Huerta Grande (Córdoba) 21-24 October, 1991
   This is basicaly the paper #1, presented in Spanish at the Annual Meeting of the Argentine Society for Biochemical Research.

5. **Genetic Organization of Junín Virus, the Etiologic Agent of Argentine Hemorrhagic Fever**
   V. Romanowski
   Invited chapter for: *"The Arenaviridae"* (M. Salvato, ed.), *"The Viruses"* series (R. Wagner & H. Frankel Conrat, eds.), Plenum press, New York (in press).
   The chapter reviews the current knowledge on the biochemistry and molecular genetics of Junín virus and contains some of the data presented in the midterm report.

6. **A simple nucleic acids amplification assay for the early and rapid detection of Junín virus in clinical specimens.**
   M.E. Lozano, P.D. Ghiringhelli, V. Romanowski and O. Grau
   Virus Research (submitted)
   The paper is based upon the results presented at meetings #2 and #3.

PERSONNEL (supported with grant funds; graduate degrees)

Pablo Daniel Ghiringhelli (DNI* 12.894.123)
Research assistant in this project since 15 May 1989.
Graduate student; is expected to submit his PhD dissertation in early 1993.

César Gustavo Albariño (DNI 14.845.746)
Research assistant in this project since 15 February 1990.
Graduate student; will probably finish his PhD project within the next two years.

Mariel Piboul (DNI 16.504.623)
Research assistant in this project during the period 15 May 1989 - 14 August 1990.
She has been enrolled in a Doctorate program, but resigned in August 1990.

Silvia Adriana Moya (DNI 5.968.287)
Secretary since 15 May 1989.

(*) National ID number in Argentina (equivalent to SS#)